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(54) Title: DETECTION OF CANCER AND ABNORMAL PREGNANCY USING MONOCLONAL ANTIBODIES SPECIFIC FOR HCG ISOFORMS

(57) Abstract

Human chorionic gonadotropin (hCG) exists in blood and urine as a variety of isoforms, one of which contains peptide bond cleavages within its beta subunit and is referred to as nicked hCG (hCGn). This hCGn isoform appears more prevalent in the urine of patients with certain malignancies and possibly in other diseases of pregnancy. The present invention is directed to two monoclonal antibodies to an isoform of hCGn isolated from a choriocarcinoma patient. Two-site immunometric assays have been developed using these antibodies, designated B151 and B152. The former exhibits good specificity for hCGn independent of the source of the hCGn, that form excreted by choriocarcinoma patients, or the form of hCGn from normal pregnancies. The latter antibody, B152, is uniquely sensitive to the carbohydrate moieties of choriocarcinoma hCG, nicked or non-nicked, since its recognition of ligand is dependent upon carbohydrate differences rather than differences in peptide bond. These two immunometric assays provide novel diagnostic tools based on direct measurement of these hCG isoforms.

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DETECTION OF CANCER AND ABNORMAL PREGNANCY USING MONOCLONAL ANTIBODIES SPECIFIC FOR HCG ISOFORMS

The present invention relates to anti-hCG monoclonal antibodies for use in detecting certain types of cancer and for detection of abnormal pregnancy. In more detail, the present invention relates to immunoassays for detection of nicked and hyperglycosylated hCG isoforms produced by certain hCG-secreting malignancies and in certain problem pregnancies.

Human chorionic gonadotropin (hCG) is the hormone produced by the placenta in pregnant women. All known pregnancy detection kits currently in use are based on the detection of hCG in the blood or urine. hCG is secreted by the trophoblast early in pregnancy and functions to maintain steroid secretion of the corpus luteum until the placenta takes over that function later in pregnancy. hCG is comprised of α and ß subunits, the ß subunit being heavily glycosylated. In addition, a variety of isoforms of hCG, including free α and ß subunits, heterodimeric (or whole) hCG with peptide bond cleavages in loop 2 of the ß fragment (referred to as nicked hCG), free nicked hCG subunits, and the ß core fragment are known. *See* S. Birken, *et al.*, 129 Endocrinology 1551-1558 (1991), S. Birken, *et al.*, 131 Endocrinology 1390-1397 (1993), and S. Birken, *et al.*, 125 Mol. Cell Endocrinol. 121-131 (1996). The nicked isoform of heterodimeric hCG has been reported to be present in blood as well as urine and is known to have much lower recognition by antibodies directed to heterodimeric hCG, its subunits, and fragments, as well as greatly reduced biological activity. S. Birken, *et al.* (1991), *supra*, A. Kardana, *et al.*, 129 Endocrinology 1541-1550 (1991), L.A. Cole, *et al.*, 129 Endocrinology 1559-1567 (1991). Hyperglycosylated hCG occurs in multiple isoforms and has been detected in blood and urine, and in cell surface membranes, and its chemical and immunological properties are summarized in M.M. Elliott, *et al.*, 7 Endocrine 15-32 (1997), hereby incorporated into this specification in its entirety by this specific reference thereto.

Detection of nicked and hyperglycosylated hCG is of interest in light of data indicating that certain hCG-secreting malignancies produce a larger proportion of these hCG isoforms. A. Puisieux, *et al.*, 126 Endocrinology 687-694 (1990), R. Nishimura,

et al., 78 Jpn. J. Cancer Res. 833-839 (1987). Detection of the glycosylated ß subunit of hCG is of interest because of its prevalence in certain problem situations of pregnancy such as preeclampsia and Down's syndrome. L.A. Cole, *et al.*, 17 Prenat. Diagn. 1180-1190 (1997), L.A. Cole, *et al.*, 18 Prenat. Diagn. 926-933 (1998). It is also of interest that the hyperglycosylated hCG appears to be prevalent in healthy pregnancies whereas those pregnancies destined for early failure have little of this hCG isoform. J. O'Connor, *et al.*, 18 Prenat. Diagn. 1232-1240 (1998).

Nicked hCG has been measured by a variety of qualitative techniques such as immunoblotting, direct isolation, and sequence analysis of hCG isoforms from urine. However, so far as is known, there are no satisfactory direct immunoassays for the nicked form of hCG, the nicked form of free hCG ß subunit, or hyperglycosylated hCG isoforms. On information and belief, all measurements to date have been conducted with subtractive assay procedures or immunoassays which include scavenger antibodies. A. Kardana, *et al.*, 38 Clin. Chem. 26-33 (1992), A. Kardana, *et al.*, 79 J. Clin. Endocrinol. Metab. 761-767 (1994), S. Rotmensch, *et al.*, 174 Am. J. Obstet. Gynecol. 609-611 (1996). So far as is known, no satisfactory assay has been developed for detecting hyperglycosylated hCG or for distinguishing between cancer-related and standard, or normal, hCG (CR 127, for example). There is, therefore, a need for an accurate assay for detection of these hCG isoforms for use in diagnosis of certain malignancies and problem pregnancies.

The present invention therefore provides a method for detecting certain hCG isoforms by providing two monoclonal antibodies, B151 and B152, each with unique specificity for hCG isoforms, for use in immunoassays for diagnosis of certain malignancies and characterization of normal and problem pregnancies. Using these antibodies, which preferentially detect nicked hCG isoforms (B151), the hCG isoform from normal pregnancies (B152), and differences between hyperglycosylated and standard pregnancy isoforms of hCG (B152), immunoassays are conducted for accurate and convenient detection of those malignancies which produce the nicked hCG isoform, Down's syndrome pregnancy, and such problem pregnancies as preeclampsia and other pregnancies which are not likely to proceed to term.

Referring to the figures, Figure 1 is a graph of the results of a liquid phase radioimmunoassay using antibody B151 (panel A) and antibody B152 (panel B).

Calibrated (by amino acid analysis), radiolabeled choriocarcinoma hCG C5 was used as tracer and solutions of pregnancy C5 chorioCG, hCG CR 127, hCG CR 127 non-nicked, and hCGn CR 127 were employed as competitors. Non-linear regression lines were plotted in logit transformed format.

Figure 2 is a graph of the results of an immunometric (two-site) assay using antibodies B152 as capture and B207 (*see infra*) as detection reagent. Binding curves are shown for various forms of hCG-related hormones as detailed *infra*. Each panel represents a separate assay in which all ligands were introduced in the same assay. Points were connected by straight lines, although regression analysis (four-parameter logistic) indicated excellent fit to logistic or sigmoidal curve-shaped model. Panels A and B represent two distinct assays with similar results.

In more detail, the present invention is based on the conception, production, identification, and characterization of two monoclonal antibodies with distinct specificity using a nicked, hyperglycosylated form of hCG purified from a single choriocarcinoma patient as an immunogen. The two antibodies, designated as B151 and B152, were selected by the use of radiolabeled C5 and CR 127 (see *infra*).

Each antibody displayed preferential binding to C5 as compared to CR 127 since this was the selection criterion. However, upon performing liquid phase immunoassays and calculating affinity constants, it was clear that these two antibodies were very different in specificity (Table I). One antibody, B151, displays significant preference in recognition of nicked hCG forms, regardless of the origin of the hCG molecule (normal pregnancy or choriocarcinoma). The second antibody, B152, displays a distinct preference for binding to choriocarcinoma hCG and is oblivious to the nicked characteristic. B152 is also partly directed towards carbohydrate differences between pregnancy hCG and choriocarcinoma hCG, regardless of the state of the nicking of the polypeptide chain. So far as is known, the only other monoclonals which discriminate between the carbohydrate moiety of hCG are antibodies to the carboxy-terminal portion of hCG-ß produced by the use of the isolated peptide bound to a carrier. A. Krichevsky, *et al.*, 135 Endocrinology 1139-1145 (1994), S. Birken, *et al.*, 122 Endocrinology 2054-2063 (1988). It is of interest that at least part of the epitope of B152 is also directed towards the carboxy-terminal portion of hCG-ß. The C5 choriocarcinoma form of hCG (described below) is the

Table I: Affinity Constants [a] by Liquid Phase Competition Assays Using C5 as Tracer Ligand

	Competitors			
Antibody	C5	Nicked hCG CR 127	Parent hCG CR 127	Nick-free hCG CR127
B151	4.4×10^8	3.8×10^8	4.2×10^7	1.3×10^7
B152	3.5×10^8	5.4×10^7	4.7×10^7	5×10^7

[a]Ka as L/M

Table II: The Specificities of the two immunometric assays developed from antibodies B151 & B152

A. Relative Cross-Reactivities of Two Site Assay Using B151 as Capture Antibody

Ligand	B207[a]	B204	B201	B108	B109	A109	CTP104
C5	100%	<[b]	<	100%	<	<	100
Nicked CR 127	100%	<	<	100%	<	<	47%
Non-Nicked CR127	12%	<	<	37%	<	<	14%
HCG β	2%	<	<	2%	<	<	<
C5 β	5%	<	<		<	<	<
HCGβ core	<	<	<		<	<	<
HLH	2%	<	<	3%	<	<	<
HLHβ	5%	<	<		<	<	<
HCG α	<	<	<	3%	<	<	<
Maximum binding	50%	0%	0%	13%	0%	0%	83%

[a]labeled detection antibodies
[b]< out of low range detection

B. Relative Cross-Reactivities of Two Site Assay Using B152 as Capture Antibody

Ligand	B207[a,c]	B204	B201	B108*	B109	A109	CTP104
C5	100%	100%	94%	42%	53%	100%	<
Nicked CR 127	10%	<[b]	<	15%	32%	64%	<
Non-Nicked CR127	7%	<	<	30%	100%	26%	<
HCG β	6%	20%	19%	11%	<	<	<
C5 β	190%	100%	100%	100%	<	<	<
HCGβ core	<1%	<	<	<	<	<	<
hLH	<1%	<	<	<	<	<	<
hLHβ	<1%	<	<	<	<	<	<
HCG α	<1%	<	<	<	<	<	<
Maximum binding	64%	2%	44%	80%	2%	14%	

[a]labeled detection antibodies
[b]< out of low range detection
[c], this particular assay format was applied in O'Connor et al 18 Prenat. Diagn 1232 (1998)

only such hCG displaying 100% hexasaccharide structure on its O-serine linked carbohydrate moieties in the ß COOH-terminal region. The monoclonal antibody produced using this hCG as an immunogen appears to be rare - the use of hCG-ß COOH-terminal region as carrier-bound immunogen resulted in production of both polyclonal and monoclonal antibodies. A. Krichevsky, *et al.* (1994), *supra*, S. Birken, *et al.* (1988), *supra*.

As noted above, monoclonals B151 and B152 were produced using hCG purified from a choriocarcinoma patient as an immunogen. Fig. 1 shows potency comparisons of liquid phase competitive radioimmunoassays of both B151 and B152 antibodies comparing competitors: (1) standard CR 127 pregnancy hCG (which has a 20% content of nicked hCG), (2) C5 chorio CG (100% nicked and hyperglycosylated), (3) nicked CG made from CR 127 by purification, and (4) non-nicked hCG derived from CR 127. The labeled ligand was C5 chorio hCG. It is apparent from Fig. 1A that B151 shows a preference for nicked forms of hCG. C5 chorio hCG or CR 127 hCGn bind with similar affinities. The slightly lower potency of CR 127 hCGn may be ascribed to its 20% contamination with non-nicked hCG. As shown in Fig. 1B, B152 only shows a preference to C5, the hyperglycosylated chorio CG. CR 127 hCGn is no more potent a competitor than nick-free CR 127 hCG.

Using the monoclonal antibodies B151 and B152, a variety of two site assays were used to detect the various forms of hCG. Table II displays the results. The molar quantity of ligand required to produce binding equal to 50% of the maximum binding achieved by C5 was determined. Crossreactivity shown in Table II as a percentage was calculated by dividing the molar quantity of the standard by the molar quantity of the other ligand at 50% maximum binding dose.

It is apparent from Table II that B151 cannot bind simultaneously with antibodies to the ß subunit and ß subunit core which are bound by monoclonals B201 and B204 (these latter monoclonal antibodies are described in A. Krichevsky, *et al.*, 123 Endocrinology 584-593 (1988)). Nor does B151 bind simultaneously with antibodies directed towards the determinant which exists in heterodimeric hCG as represented by antibody B109 (*see* A. Krichevsky, *et al.* (1988), *Id.*). In contrast, a general beta antibody which binds to the most common and potent hCG antigenic site such as B108 or B207 (*see* A. Krichevsky, *et al.*, 2 Endocrine 511-520 (1994)) binds

well to both B151 and B152 antibodies. B152 binds simultaneously to all antibodies tested except those to the ß COOH-terminal region (CTP). A. Krichevsky, *et al.*, 134 Endocrinology 1139-1145 (1994). By contrast, B151 binds well to hCG already bound to CTP antibodies, indicating that it may represent a newly revealed hCG epitope which only exists on nicked hCG.

Using B152 as capture and B207 or B108 as detection antibody produces an assay which measures all normal pregnancy forms of hCG (both intact and nicked and ß subunit) to a similar extent but prefers binding to the form of hCG or ß subunit to its C5 immunogen. This assay does not prefer nicked forms of hCG but hyperglycosylated forms such as C5. B152 and CTP104, as well as several other monoclonal antibodies to the COOH-terminal region of hCGß, cannot bind simultaneously to C5, implying that this region is part of or very close to the epitope of I-152. These data taken together with the apparent B152 preference for hyperglycosylated hCG implies that the carbohydrate of the CTP region may be part of the B152 epitope.

The possibility that the CTP region is part of the epitope to which B152 binds was tested by comparing the relative binding potencies of a series of isoforms of hCG in a two-site immunoassay with monoclonal antibody B207-I^{25}. The results are shown in Fig. 2 and the method is described in detail below. The isoforms of hCG utilized were (1) C5, the choriocarcinoma hCG which is glycosylated and has hexasaccharide carbohydrate structures in its beta COOH-terminal region, (2) 814, which is non-nicked hCG prepared from the hCG reference preparation CR127 derived from normal, late first trimester hCG, (3) 813, which is nicked hCG (80% nicked) produced from CR127 by hydrophobic chromatography, (4) M4 hCG, which is nicked hCG with regligible hyperglycosylation, and (5) M1A hCG, which is non-nicked and not significantly hyperglycosylated, but which is missing 80% of the hCGß COOH-terminus. The B152 two-site assay prefers to bind to C5, its immunogen, but shows nearly equal recognition of both 813 and 814, nicked and non-nicked hCG of normal pregnancy, confirming that B152 does not display significant preference for the nicked form of hCG but rather for the form with carbohydrate differences. This lack of preference is also confirmed by the potency of M4, which is also 100% nicked as is C5, but which is not hyperglycosylated and displays a potency similar to CR 127 hCG

whether nicked or non-nicked. M1A is the least potent ligand and is the only one missing most of its beta COOH-terminal peptide, confirming the role of this region in the B152 epitope.

To summarize, antibody B151 prefers binding to nicked forms of hCG, regardless of the origin of the hCG molecule (normal pregnancy or choriocarcinoma). This preference is shown by similar binding to both preparations of nicked hCG, C5 and nicked CR127. The latter preparation is not hyperglycosylated as is C5. Nick-free CR127 has the lowest affinity to this antibody as would be expected for a nick-directed antibody. This finding led to the conclusion that B151 was directed towards an epitope dependent upon peptide bond cleavages in hCG beta subunits while binding to B152 was not affected by peptide bond cleavages within this loop. Since the main difference between C5 hCG and CR 127 hCGn is the hyperglycosylation of the former, it was concluded that B152 was a carbohydrate-directed antibody. When B151 is used as capture antibody and virtually any general beta antibody as detection antibody in a two site assay (see Table II), little cross-reaction with hLH is observed. B151 cannot bind simultaneously with antibodies that are directed chiefly to the hCG beta core region (such as B201 and B204), nor can the antibody bind with antibodies directed to heterodimeric hCG such as B109 or A109, but it can bind at the same time as antibodies to the beta COOH-region. B151 therefore represents a new hCG epitope revealed after nicking of the beta subunit.

The second antibody, B152, preferentially binds with hCG with the type of carbohydrate modifications prevalent in choriocarcinoma CG C5 regardless of the state of the nicking of the polypeptide chain. Antibody B152 appears to be specific for the hCG beta COOH-terminal region. This specificity is shown by failure of a monoclonal antibody to bind simultaneously with beta COOH terminal antibodies such as CTP 104. In two site assays with B152, it was shown that hCG isoform M1A, which is missing most of its COOH-terminal region, binds poorly to B152.

Each antibody has a different application in accordance with its specificity. Using the monoclonal antibodies B151 and B152, immunoassays are designed for detection of hCG isoforms for use in the diagnosis of certain malignancies and characterization of normal and abnormal pregnancy. B151 permits formulation of direct assays for nicked hCG which have been applied in studies of early pregnancy.

These measurements have diagnostic applications for certain cancers and in the detection of Down's syndrome. The B152 antibody is used in assays which detect differences in the carbohydrate portion of hCG. Major potential applications of this antibody include detection of Down's syndrome and recognition of pregnancies destined for early pregnancy loss.

Specifically, body fluid samples such as blood from the patient are tested for the presence of the nicked hCG isoforms characteristically produced during Down's syndrome pregnancy and certain malignancies with B151 and for the presence of the hCG isoforms characteristically produced during problem pregnancies such as Down's syndrome and preeclampsia. Further, an assay utilizing B152 is used to quantify the carbohydrate variant characteristically produced during healthy pregnancies since pregnancies which are likely to fail early have proportionately little of this hCG isoform. Such immunoassays are known to those skilled in the art and need not be described in detail here. It is sufficient to describe such immunoassays as assays for the detection of the hCG isoforms characteristically produced by these various conditions and to note that such assays may be conducted by, for instance, immobilizing the hCG and labeling the antibodies for detection of the antibodies or by the immunoassays described herein.

The present invention can be better understood by those skilled in the art by reference to the following, non-limiting examples describing specific preferred embodiments of the invention.

Example 1
Immunization of Mice

Mice were immunized intraperitoneally with choriocarcinoma hCG preparation C5 (Kardana, A., *et al.*, 129 Endocrinology 1541-1550 (1991)) diluted in saline (600 µg/ml) and mixed 1:1 with Freund's adjuvant. After three consecutive immunizations (50 µg/mouse) at 3-4 week intervals, animals were rested for three months and then given an intraperitoneal booster immunization. P.H. Ehrlich, *et al.*, 8 Am. J. Reprod. Immunol. Microbiol. 48 (1985). Ten days after the last booster, the sera from the mice were tested for binding in the liquid phase radioimmunoassay described *infra* to both iodinated CR 127 hCG and to iodinated C5 hCG. Nick-free HCG (814) and nicked hCG (813) were prepared from pooled urine standard hCG (batch CR127) by

hydrophobic chromatography. Birken, S., *et al.*, 133 Endocrinology 1390-1397 (1993).

Example 2
Liquid Phase Assays

Liquid phase assays were performed using a solution of 80 µl 0.3M PBS with 0.02% sodium azide, 50 µl tracer, 20 µl normal mouse serum, 50 µl 1% horse serum free of gamma globulin for titrations (when competition studies were performed, 50 µl of competitor solution were substituted to generate dose/response or Scatchard curves), 100 µl of diluted sera followed by incubation for one hour at 37°C, then one hour at ambient, then centrifuged, supernatant aspirated, and pellets counted. Affinity constants were calculated by Scatchard plots as described in P.H. Ehrlich, *et al.* (1985), *supra*, G. Scatchard, 51 Ann. NY Acad. Sci. 660 (1949), both references being incorporated herein in their entirety by this specific reference thereto.

The mice whose antisera had the greatest discrimination between binding of radiolabeled C5 and radiolabeled CR 127 hCG were sacrificed and their spleens were used for hybridoma production by the methods described in A. Krichevsky, *et al.* (1988), *supra* and P.H. Ehrlich, *et al.* (1985), *supra*, both references being hereby incorporated in their entireties by this specific reference.

Example 3
Two Site Assays

Two site assay testing was conducted by the method described in N.J. Ellish, *et al.*, 11 Human Reprod. 406-412 (1996), which reference is also incorporated into this specification in its entirety by this specific reference. Briefly, Immulon 4™ microtiter wells are coated with capture antibody (as described above) at a titer determined to provide the best combination of sensitivity and range. The antibody in coating buffer (sodium bicarbonate 0.2M, pH 9.5, 200 µl/well) is incubated overnight at 4°C, the solution aspirated and the plates washed (wash buffer was PBS with 0.05% TWEEN 20™). The plates were then blocked with 1% bovine serum albumin (BSA) in PBS overnight at 4°C and again aspirated and washed. Standards, clinical samples and controls were added to the coated wells (200 µl/well), incubated 24 hrs at 4°C, after which well contents were aspirated, washed (5X) and labeled antibody (either I^{25} labeled antibody (50K-100K CPM/well) or peroxidase-labeled) added and then again incubated 24 hrs at 4°C. The wells were aspirated, washed (5X), and counted by γ-

counter (Packard Instruments COBRA, Meridien, CT) or by absorption spectrophotometry as appropriate. A cubic spline curve was generated for standard values and sample values were interpolated from that curve. Regression lines and all graphs were created using SIGMAPLOT™ 4.01 from SPSS Software (Chicago, IL). Linear regression analysis of immunopotency (Table II) as compared to each of the carbohydrate differences of the hCG isoforms (Table I) was accomplished with INSTAT 1998 (GraphPad Software, Inc., San Diego, CA).

* * * * *

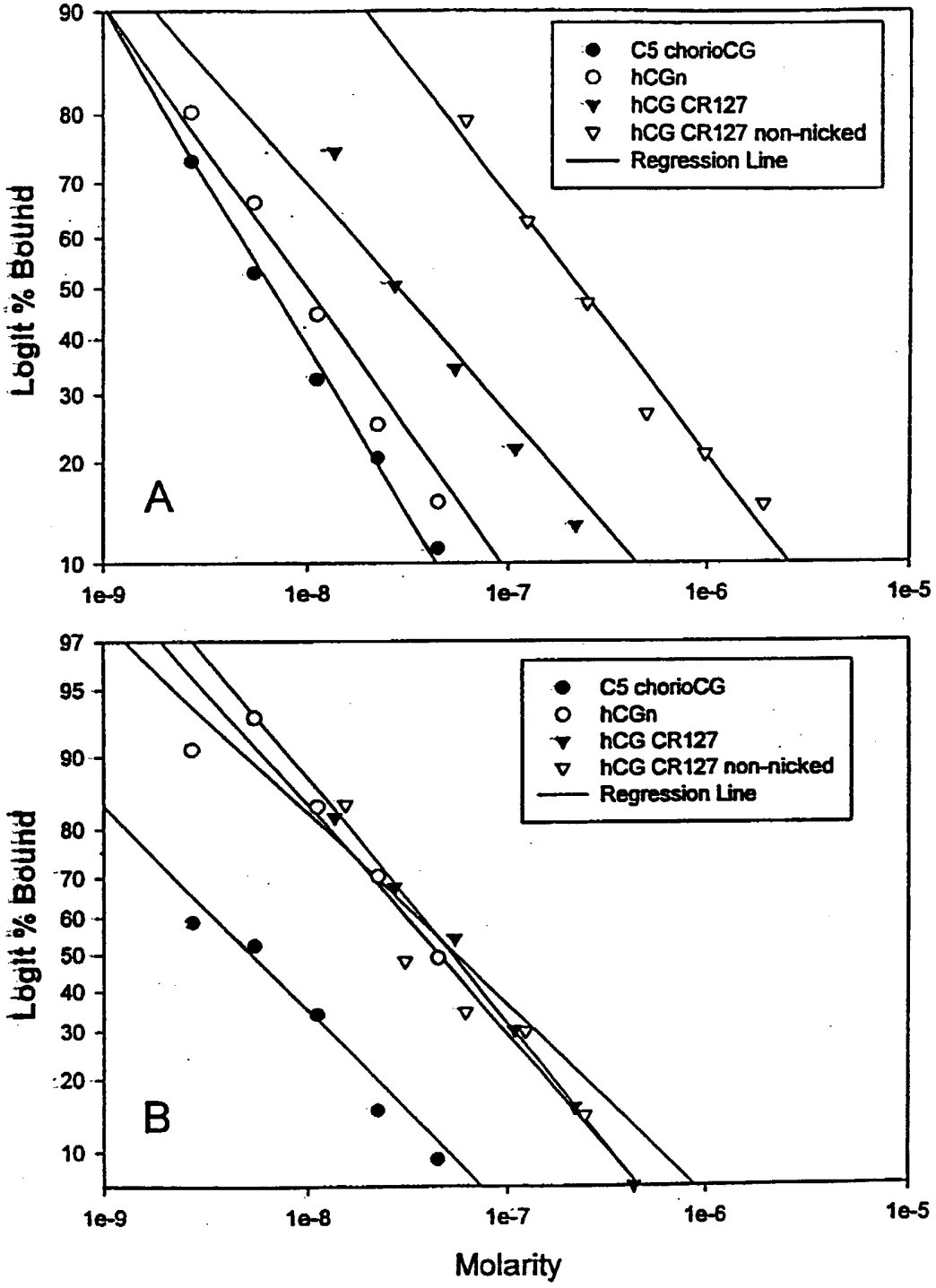
Although described in terms of the above specific examples, those skilled in the art who have the benefit of this disclosure will recognize that these examples are provided to exemplify the invention and not for the purpose of limiting the invention to the specific examples described. To the contrary, the skilled practitioner will recognize that changes may be made in individual elements and/or steps in these specific embodiments without changing the manner in which these embodiments function to achieve their intended result(s). All such changes are intended to fall within the scope of the following claims.

What is claimed is:

1. Monoclonal antibody B151.

2. Monoclonal antibody B152.

3. The use of monoclonal antibodies B151 and B152 for detection of hCG isoforms for diagnosis of malignancies and problem pregnancies.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hCGn
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hCG CR127 non-nicked
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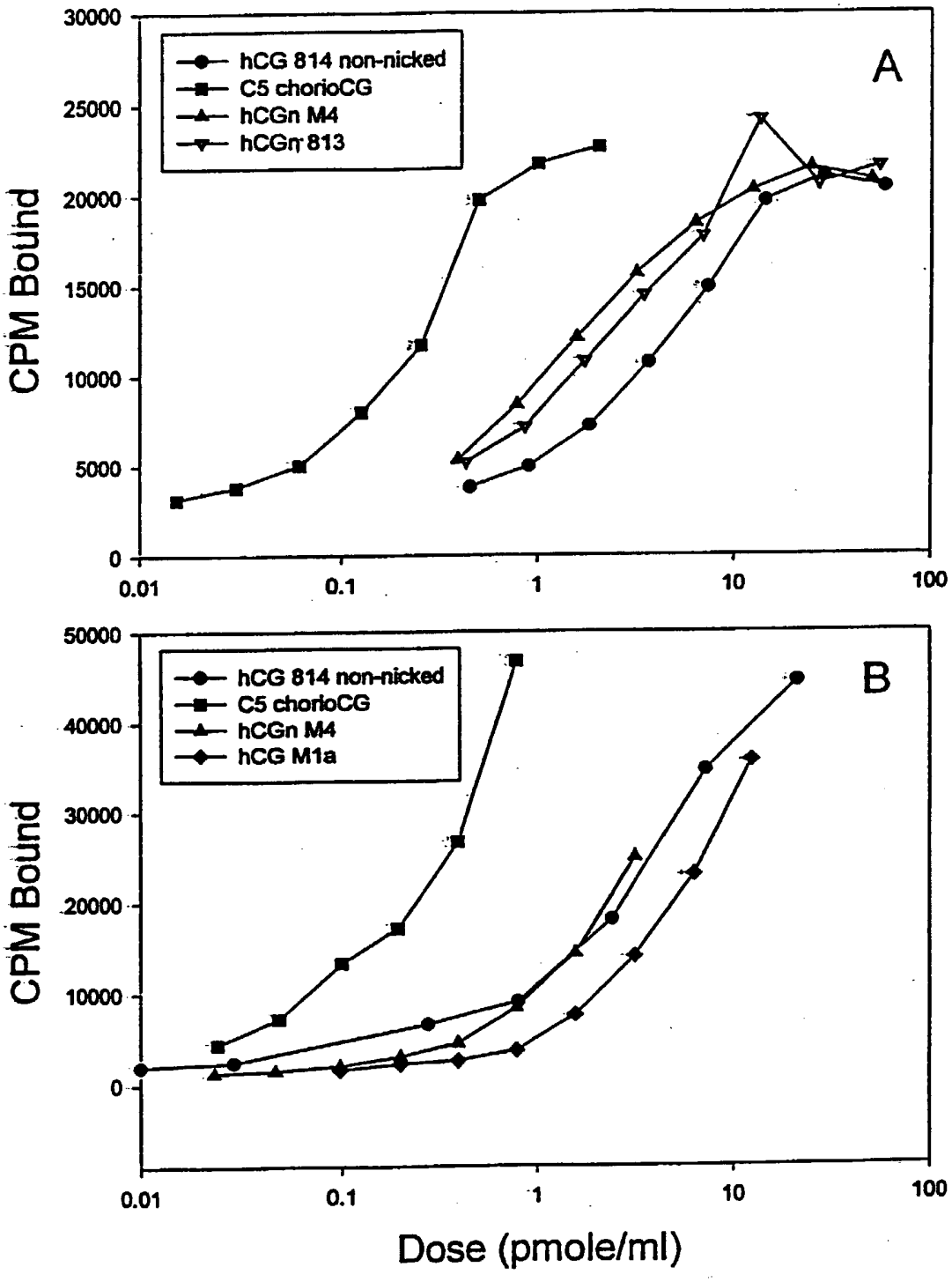
1e-5

FIG. 1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0.01
0.1
1
10
100
B
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C5 chorioCG
hCGn M4
hCG M1a
CPM Bound
50000
40000
30000
20000
10000
0
0.01
0.1
1
10
100
Dose (pmole/ml)

FIG. 2